Green Flourescent Protein Expression in Bone Marrow Derived Mesenchymal Stem Cells of Immunocompotent and Aythmic Nude Rats

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ABSTRACT

Concerns exist regarding potential differences in transgene product expression following gene delivery in immunocompetent versus immunocompromized animal models. To address this question *in vitro*, this study evaluated expression of green flourescent protein (GFP) in bone marrow-derived mesenchymal stem cells (BMDMSC) from immuncompetent and athymic nude rats following GFP gene delivery using a first-generation adenoviral vector. Cell types used were from 2-4-week old Wistar (Wstr) rats and 10-week-old NIH-*rnu* nude rats (Nude) of both sexes, and a BMDMSC from Lewis rats (Tul). Intensity and duration of GFP expression were documented every 48 hours in live cells using an *in vivo* imaging system with a cooled charge-coupled device camera. Intensity was measured in flux (photons/cm^2/second/steradian). Expression was compared between cell types in cells in monolayer and three-dimensional alginate culture conditions. Flux values from days 0, 7, 12, and 22 were used for statistical comparisons. There was no significant difference in GFP expression between groups (immunocompetent *versus* immunocompromized). Rat breed and immune status had no effect on GFP expression ($P > 0.8$, monolayer; $P > .60$, alginate). Background flux was 2.50×10^6 for cells in monolayer culture and 1.42×10^6 for cells in alginate constructs. The AdGFP-transduced cells had 1800-fold greater expression, when compared with background values, in monolayer culture, and 525-fold greater expression in alginate constructs. Cells cultured in monolayer showed significantly greater expression compared to those in alginate constructs ($P < 0.05$). These findings will provide valuable information for selection of optimal target cells for gene delivery using Ad vectors, and may alleviate concerns regarding potential differences in transgene expression between

and immunocompromized and immunocompetent animal models.

INTRODUCTION

Impaired fracture healing and osteoarthritis are sources of morbidity in dogs[1], rats, horses, humans[2] and other species and the incidence of both increases with age. Osteoarthritis (OA) is a disease that affects the joints of the body causing pain and disability. It is a disease that lacks an effective treatment and mostly affects the hips, knees and distal interphalangeal joints of the hands[3]. Fractures can occur in any bone of the body, near joints or in the long bones. Approximately 10-15% of fractures in human patients will become delayed or non-unions[2], i.e. they will not heal within a reasonable time period, based on clinical criteria. In both OA and delayed and non-union fractures, augment of biologic processes is indicated for the alleviation of pain and disability.

One of the most promising potential therapies is gene therapy. Reporter genes are commonly used to confirm the function of gene delivery systems. Prior to the development of potentially therapeutic transgene constructs, it is essential that the efficiency of new gene delivery systems be validated by the use of reporter genes. Green fluorescent protein (GFP), found in nature in the jellyfish (*Aquoria victoria*), is a widely used, reliable reporter gene. Numerous vector systems have been evaluated for the delivery of cDNAs of interest to cells, in both *in vivo* and *ex vivo* gene delivery systems. In this study, a recombinant human adenovirus was chosen because this vector has a high transduction efficiency, is easy to propagate with high titers at a reasonable cost, and it can transduce both dividing and ordinary cells[4].

Bone morphogenetic proteins (BMPs) are a family of growth factors shown to improve fracture healing by inducing osteogenic differentiation of mesenchymal progenitor cells via the mechanism of endochondral ossification. The most widely used and studied BMPs are BMP-2 and BMP-7[6]. They also been successfully used in clinical settings for spine fusion and management of recalcitrant non-union fractures of the tibia[7,8].

Bone marrow derived mesenchymal stem cells (BMDMSC) promote bone healing by their ability to differentiate into osteoblasts and chondrocytes, as well as other mesenchymal cells[9], and as such, have been used extensively as gene delivery targets[10]. Immunodeficient animal models have been used to evaluate various gene therapy systems *in vivo*, providing the obvious advantage of the opportunity for allograft and xenograft cell transplantation. However, questions exist concerning the translational research potential of such models. Regardless of these concerns, immunocompetent animal models will continue to serve as valuable screening tools for the preliminary assessment of gene therapy techniques prior to more rigorous testing in immunocompetent animals.

One of the delivery vehicles which has been well-studied to date is alginate, a linear copolymer derived from the kelp plant (*Macrocystis pyrifera*). Alginate is economical to purchase and store. It also has the important advantage of ease of handling, which becomes relevant when looking toward translational applications.

The purpose of the study reported here was to evaluate and compare GFP expression in BMDMSC from immunocompetent and athymic nude rats. Our hypothesis was that GFP expression in BMDMSC from athymic nude rats and those from Wistar (immunocompetent) rats would differ in neither intensity nor duration of transgene expression. Our specific goal was to evaluate GFP expression by documenting duration

and intensity of fluorescence detected in cells from both immunocompetent and athymic rats by imaging of transgene product production in live cells. Bioluminescence imaging has been used as an acceptable evaluation in other models[11]. Determining the differences in expression of GFP between these two cell types will provide valuable information to be used in the selection of optimal target cells for gene delivery using Ad vectors.

MATERIALS AND METHODS

Cell Culture

Bone marrow derived mesenchymal stem Cell types used were from 2-4-week old Wistar (Wstr) rats and 10-week-old NIH-*rnu* nude rats (Nude) of both sexes, and a BMDMSC from Lewis rats* (Tul). Cells were expanded in monolayer culture in using Tulane Complete Culture Medium. Media contained alpha-MEM with L-glutamine supplemented with 20% fetal bovine serum (FBS), additional L-gluatmine (200mM in 0.85% NaCl; final concentration 2mM), penicillin, (final concentration 100 units/ml and 100µ), and streptomycin (final concentration 100µg/ml).

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Monolayer

Cells were placed in 48 well clear plates with 1000 cells per well when seeded at Day -1 (Day 0 = day of transduction). Cells were transduced for 2 hours with an adenoviral-GFP transgene construct containing a first generation adenoviral vector (AdGFP)[12]. After 2 hours all virus was aspirated off and replaced with 700µl of Tulane CCM. Starting at

Day 0, the fluorescence of the cells was measured using a commercially available *in vivo* imaging system (IVIS®, Xenogen Corporation, Alameda, CA).

Alginate

Cells were plated at a seeding density of 500,000 cells per well in 75mm^2 plates and incubated at 37°C, 5% CO_2, until 75% confluent. Three plates were transduced with an adenoviral-GFP transgene construct containing a first generation adenoviral vector (AdGFP)[12] at a total multiplicity of infection (MOI) of 10,000:1 for two hours at 37°C. All media was aspirated from cells and replaced with 6ml trypsin-EDTA (1x) for 15 minutes 37°C, 5%CO_2. Trypsin action was stopped by adding 6ml of Tulane CCM media to each dish. Cells were then centrifuged at 1400rpm for 10 minutes. Media was aspirated off and cells were rinsed with 6ml GBSS and centrifuged at the same speed and time. Using a 1cc syringe and 20 gauge needle cells were re-suspended in 200µl of 1.2% Na alginate. A pipette was then used to measure 50µl of the mixture for one bead and placed in $CaCl_2$ for 10 minutes. Beads were then rinsed three times in 0.9% NaCl and placed in a 96 well plate, one bead per well with 75µl Tulane CCM. The cells were then scraped and made into alginate beads, containing approximately $5.0x10^5$ cells per alginate bead. The beads were then placed in a 96-well black plate containing clear well bottoms with Tulane CCM in each well. Fluorescence imaging began at Day 0.

Histological Staining

Staining for alkaline phosphatase production was performed using a commercially available kit per the manufacturer's instruction (Sigma kit No. 85, Sigma-Aldrick, St. Louis, MO). Staining for mineral using the performed using the von Kossa method.

Statistical Analysis

Unpaired t-tests were done to compare expression at Days 0, 7, 12, 22. . A one-way ANOVA was performed to determine the difference of GFP expression across the three different cell types, Nude, Tulane, and Wstr cells. Individual tests were run for the control (i.e. untransduced), transduced AdGFP cells, in monolayer and the alginate construct.

RESULTS

There was no significant difference in GFP expression between groups (immunocompetent *versus* immunocompromized). Rat breed and immune status had no effect on GFP expression ($P > 0.8$, monolayer; $P > .60$, alginate). Flux values, expressed as a ratio of median expression in AdGFP-transduced cells to background levels, were as follows: Monolayer Day 0 -7792/1; Day 7 – 2634/1; Day 12 – 2592/1; Day 22 – 3819/1;Alginate Day 0 – 2304/1; Day 7- 529/1; Day 12 – 488/1; Day 22 421/1. Background flux was 2.50×10^6 for cells in monolayer culture and 1.42×10^6 for cells in alginate constructs. The AdGFP-transduced cells had 1800-fold greater expression, when compared with background values, in monolayer culture, and 525-fold greater expression in alginate constructs. Cells cultured in monolayer showed significantly greater expression compared to those in alginate constructs ($P < 0.05$)

Figures 1 and 2 show expression of cells in both monolayer and alginate construct at Day 0.

Figure 1.

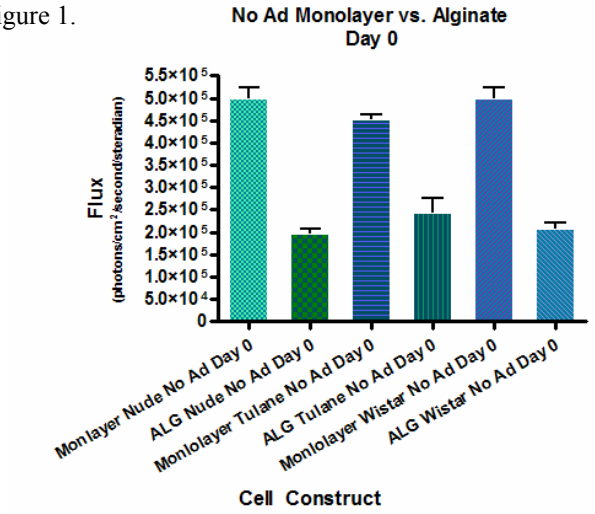
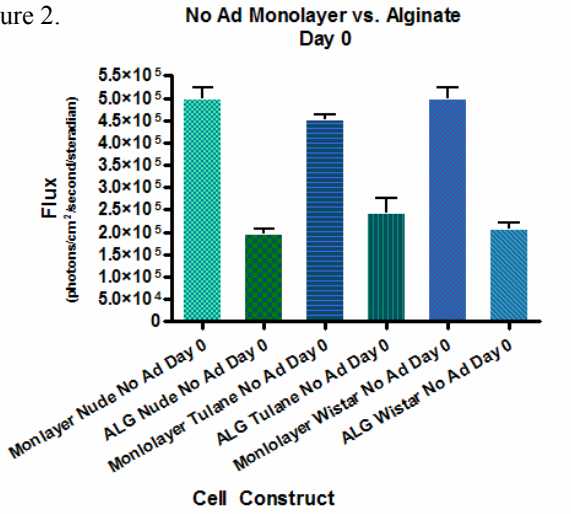


Figure 2.



Figures 3 and 4 show expression of cells in both monolayer and alginate construct at Day 22.

Figure 3.

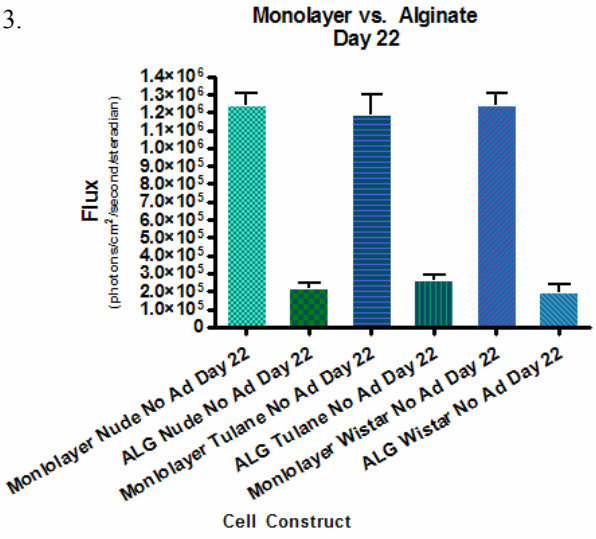
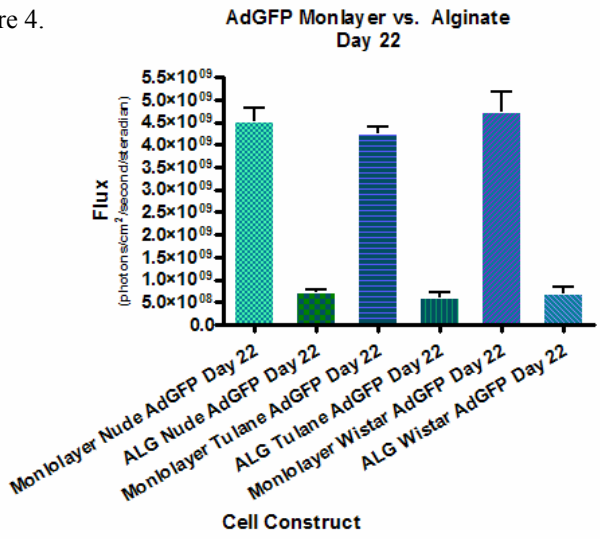


Figure 4.



DISCUSSION

We conclude that in this *in vitro* model of transgene expression in monolayer and three-dimensional alginate cultures, there is no difference in transgene expression between cells from immunocompetent animals and those of from immunodeficient nude rats.

Cells in monolayer had greater transgene product expression than those in alginate. This may be due to a decreased ability for nutrient exchange between the culture medium cells in this system. Cells in monolayer have free access to media and therefore can transport the necessary molecules for protein production.

However, this is only one factor that could have caused the lack of expression. The cells themselves could have suffered damage from being frozen and thawed that prevented the virus from transduction.

These results provide us with more information with which to pursue in vivo studies and decrease the concern regarding potential impairment of transgene expression in cells from immunodeficient animals. It has shown that adenovirus can be used as a successful vehicle to deliver genetic material into cells and express protein for an extended period of time. This can lead to further study using therapeutic genes to promote the expression of protein in multiple tissues.

The fact that there was no statistical difference between the immunocompotent and immunodeficient cell lines leads to the idea that the immunocompotent cells may be used to delivery vehicles themselves, such as in the alginate construct. Further study could also be done using different scaffolding to determine a better source to introduce

desired protein *in vivo*. Overall it shows potential for the use of gene therapy to aid in the curing/prevention of illness and disease.

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